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## **Does the anticipatory behaviour of chickens communicate reward quality?**

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## 1    **ABSTRACT**

2    The anticipatory behaviour of animals has been credited with enabling scientists to more closely infer  
3    what an animal wants. From a welfare perspective, this knowledge could improve how we care for  
4    animals under our management, as information about how animals prioritise rewarding items may  
5    guide how we allocate resources effectively. Our goal was to determine if behaviour in anticipation  
6    of different types of reward was differentially expressed. We investigated whether certain behaviours  
7    were characteristic of anticipation of both food and non-food rewards, and whether signals indicating  
8    rewards led to increased activity levels. Twelve laying hens experienced a Pavlovian conditioning  
9    paradigm using sound cues to signal the availability of two different food rewards (mealworms,  
10    normal food), one non-food reward (a container of mixed soil and sand substrate suitable for foraging  
11    and dustbathing (Dusty substrate)) and a sound-neutral event, which was signalled by a sound, but no  
12    reward was given. A muted-neutral treatment (no reward and no sound cue) controlled for any  
13    specific behaviour as a result of the sound cues. Behavioural responses and the number of transitions  
14    between behaviours were measured during a 15 second anticipatory period, before birds accessed  
15    rewards in an adjoining compartment by pushing through a door. These responses and latency to  
16    access the rewards were analysed using linear and generalised linear mixed models. Differences in  
17    pushing and pecking at the door (frequency: Dusty substrate 4.87<sup>a</sup>, Mealworm 3.18<sup>b</sup>, Normal Food  
18    2.23<sup>b</sup>, Sound Neutral 0.30<sup>c</sup>, Muted Neutral 0.03<sup>d</sup>,  $\chi^2(4)=228.99$ ,  $p<0.001$ ), standing (not walking)  
19    (duration (s): Sound Neutral 9.92<sup>c</sup>, Muted Neutral 7.49<sup>bc</sup>, Normal Food 7.39<sup>bc</sup>, Mealworm 7.05<sup>b</sup>,  
20    Dusty substrate 3.06<sup>a</sup>,  $\chi^2(4)=36.28$ ,  $p<0.001$ ), reflected the perceived value of the rewards, with  
21    birds appearing to be more motivated to access the Dusty substrate compared with the food rewards.  
22    Rewarded sound cues elicited increased transitions between behaviours, compared with neutral events  
23    (Dusty substrate 10.16<sup>a</sup>, Mealworm 10.13<sup>a</sup>, Normal Food 9.22<sup>ab</sup>, Sound Neutral 7.89<sup>bc</sup>, Muted Neutral  
24    6.43<sup>c</sup>,  $\chi^2(4)=72.05$ ,  $p<0.001$ ). The sound-neutral treatment induced increased head movements,  
25    previously associated with anticipation of rewards (duration (s): Sound Neutral 1.58<sup>b</sup>, Muted Neutral  
26    0.58<sup>ab</sup>, Normal Food 0.48<sup>a</sup>, Mealworm 0.27<sup>a</sup>, Dusty substrate 0.00<sup>a</sup>,  $\chi^2(4)=25.56$ ,  $p<0.001$ ). Latency  
27    to access rewards conveyed the relative value of rewards (Dusty substrate 7.30<sup>a</sup>, Mealworm 10.06<sup>ab</sup>,  
28    Normal Food 16.53<sup>b</sup>,  $\chi^2(2)=10.88$ ,  $p=0.004$ ). Our experiment indicates that, under certain conditions,

hens increase their activity levels (behavioural responses and transitions) in anticipation of rewards. Importantly, we demonstrate that this response is not food specific, but rather a general response to both food and non-food rewards. This outcome extends our knowledge of reward-related anticipatory behaviour, and of how hens rank rewards of contrasting incentive value, which may have implications for the methods and environments applied to improve the welfare of laying hens in managed systems.

**Keywords:** Anticipatory behaviour, conditioning, rewards, dustbath, chickens.

## 1. INTRODUCTION

An important goal of farmers, welfare scientists, and those who create the legal frameworks for food production, is to find an optimal environment that balances production and welfare. One key aspect of animal welfare is to provide an environment in which animals' wants and needs can be satisfied (Dawkins, 2012). Measuring what animals want, therefore, is crucial to developing animal management systems that provide good welfare. One such method is to investigate the anticipatory behaviour of animals in order to examine how they perceive potential stressors or rewards.

A reward is defined as anything that an animal will work for (Rolls, 2000), in contrast to a punisher, which is defined as a stimulus that decreases the probability of actions on which it is contingent (Rolls, 2005). Neuroscience experiments have revealed that the period between a signal indicating the arrival of a reward and the actual presentation of the reward is when behavioural activity correlates with pleasure-based (dopaminergic) activity in the brain (Berridge, 1996). Various accounts of anticipatory behaviour have described it as 'preparatory behaviour' (Matthews et al., 1996), or goal-directed behaviour (Wit and Dickinson, 2009), leading to and facilitating consummatory behaviour (Berridge, 1996). Importantly, this state of "wanting" can be directly observed, potentially providing a means of measuring how animals prioritise one reward over another (Dawkins, 2012). Anticipatory behaviour may also demonstrate how sensitive animals are to a reward (Spruijt et al., 2001; van der Harst et al., 2003), delivering insight into their current welfare state, although caution must be used as

the correlation between choices animals make and welfare indicators is not always clear (Nicol et al., 2009).

In a rewarding environment, animals often exhibit appetitive and consummatory behaviour around certain resources (Spruijt et al., 2001). Anticipation requires the ability to make contingent the association that one event precedes another (Greiveldinger et al., 2011), and, therefore, in order to investigate behaviour in anticipation of rewards, one approach is to train animals to associate a stimulus with the arrival of a particular reward. Presentation of the stimulus should subsequently elicit a behavioural response which is reward-related, and therefore may indicate excitement or arousal. This type of research has enabled scientists to characterise reward-related anticipatory behaviour in chickens, horses, pigs and lambs (Moe et al., 2009; Peters et al., 2012; Reimert et al., 2013; Anderson et al., 2015). Elicited behaviours vary, with increased activity and more frequent transitions between different behaviours being characteristic of some animals such as pigs (Imfeld-Mueller and Hillmann, 2012) horses (Peters et al., 2012) and mink (Vinke et al., 2004), while cats appeared to show a decrease in activity (Bos et al., 2003).

Previous research has produced some conflicting results in terms of characterising anticipatory behaviour in chickens. Kostal et al. (1992) found that broilers showed increased walking prior to their scheduled feeding time, which they interpreted as appetitive foraging behaviour shown in anticipation of the arrival of food. This increase in activity is reminiscent of the activity shown by mammals as described above. However, in a study by Zimmerman et al. (2011), hens showed no increase in locomotory activity in anticipation of a palatable food reward (mealworm), but increased their activity prior to a negative event (being squirted with water) and during a control treatment. The authors concluded that locomotory activity was not a good indicator of anticipation of a positive event in chickens. In other studies, Moe et al. (2009; 2011; 2013) defined anticipatory behaviour in laying hens, based on a description previously made by Buijs et al. (2006), as arousal-related behaviours, performed in sequence, specifically “standing still or taking slow steps, with legs, body and neck stretched upwards and eyes open, and frequent head movements.” After part of this display was

attenuated by administration of a dopamine antagonist, the authors concluded that frequent head movements “in any direction” were under dopaminergic control (Moe et al., 2011), and suggested that head movements may represent the activation of the dopaminergic reward system in hens. However, the behaviour described was characterised by slow and measured movements rather than by the increased locomotory activity seen in the study done by Kostal et al.(1992).

Thus, the research on chickens has not been able to definitely clarify whether chickens demonstrate high or low activity levels in anticipation of rewards. In addition, there is ambiguity around the contexts that elicit head movements; in the study by Zimmerman et al.(2011), the negative event elicited an increase in the frequency of head movements compared with the positive and the neutral event, a result which seems to contradict the proposal by Moe et al.(2011) that head movements represent activation of the internal reward system in hens. Indeed, Zimmerman et al. (2011) concluded that head movements could express anticipation of a negative event in general, or of their specific negative event, and they also suggested that head movements could indicate increased vigilance, or an effort to locate the source of the sound cue. They also found that comfort behaviour was associated with anticipation of a positive event, and suggested that this behaviour may reflect positive affect in laying hens. These differences in behavioural expression between studies could result from variations in experimental procedure; the study by Kostal et al.(1992) observed broiler chickens in their home environment; the experiment conducted by Zimmerman et al. (2011) recorded the behaviour of laying hens in an experimental anticipation compartment connected to a reward compartment; and Moe et al. (2009; 2011; 2013) observed singly housed laying hens, using an automated system to deliver rewards.

The conditioning paradigm studies cited above focussed on using food as an unconditioned stimulus to induce anticipatory behaviour. Moe et al. (2009) investigated whether different trace intervals following a conditioned stimulus would induce differential displays of behaviour in anticipation of a mealworm reward . In a subsequent study, again using mealworm as the reward, Moe et al. (2011) investigated whether a dopamine D2-like receptor antagonist would decrease displays of anticipatory

behaviour without affecting consumption of the reward. Their next study explored whether anticipatory behaviour reflected the incentive value of two food rewards (mealworm and whole-wheat (Moe et al., 2013), and a further study compared the behaviour of domestic fowl in anticipation of a mealworm reward with that of the Red Jungle Fowl (Moe et al., 2014). Zimmerman et al. (2011) also used mealworms as their reward, when comparing behaviour in anticipation of a positive (rewarding) or a negative event.

These studies illustrate a lack of certainty over what constitutes reward-related anticipatory behaviour in chickens - whether it is characterised by an increase in activity or by slow steps, accompanied by head movements. In addition, it is not known how chickens behave in anticipation of non-food rewards, such as prized environmental resources like a substrate suitable for dustbathing or perches (Olsson and Keeling, 2000; 2005).

Therefore, the goal of our study was to characterise the behaviour of laying hens in anticipation of different types of reward, and, more specifically, to investigate whether hens differentially express the quality of rewards in their behaviour. In order to provide more conclusive evidence of the general types of behaviour we should expect to see when hens are in a state of “wanting”, we also deemed it necessary to investigate whether anticipatory behaviours shown in our experiment were simply food-related or could be generalised to other rewarding items. To achieve this, we experimentally induced anticipation of these rewards using a Pavlovian conditioning paradigm. We used items that are known to be rewarding to hens, including two food items (mealworm and normal food) and a tray containing a topsoil/sand substrate suitable for dustbathing (Bruce et al., 2003; Olsson and Keeling, 2005).

We predicted that the frequency and duration of behaviours in response to sound cues signalling the rewards would reflect the perceived quality of the different rewards, and that cues signalling food rewards would induce a higher intensity of behavioural expression. We expected that the latency to access the rewards, as a proxy of motivation, would provide a further indicator of the quality of the reward as ranked by the birds. We also reasoned that, if anticipation of rewards elicits appetitive

behaviour and ultimately consumption of the reward, then hens would show behaviour that indicates readiness for accessing the reward. Therefore, as our hens were able to see the reward location and had to push through a door to reach the rewards, we expected that they would demonstrate increased activity when rewards were signalled.

## **2. METHODS**

### **2.1 Subjects and Housing**

Twelve ISA Brown hens, approximately 18 weeks old, were obtained from the University of Queensland's poultry unit. The hens were housed in groups of three birds in pens measuring 266 x 266 x 133cm (height). The floor of the home pen was shredded rubber chip, and each pen contained a perch at height of 41cm, (length 149cm, width 119cm), and two nest-boxes (35 x 40 x 45cm (height)). Food (standard layer pellets) and water were available ad libitum in the home pens. The housing had natural light as well as artificial light (on between 06:00 and 18:00h). There was no temperature control, however all experimental work was conducted between 08:00 and 12.30 to standardise the conditions. Hens were individually identifiable to the experimenter based on plumage colouring, marking and comb size, avoiding the need for individual marking or ringing. The methods used in this study were approved by the University of Queensland Animal Ethics Committee (Ref. SVS/314/12)

### **2.2 Treatment Groups**

Hens were subjected to a Pavlovian conditioning paradigm as used by Zimmerman et al. (2011) and Moe et al. (2009). In our experiment, an initially neutral stimulus (conditioned stimulus, CS) was repeatedly paired with the presentation of one of three different rewards (Mealworm, Normal food, or Dusty substrate) or a Sound Neutral (SN) event (an empty compartment) which served as the unconditioned stimuli (US). We used different sound cues for the conditioned stimuli, all of five seconds duration: 'ring' (ringing of an old fashioned telephone), 'beep' (an alarm-clock style beep) 'buzz' (a buzz sound as in a game show) and 'horn' (an old-fashioned car horn sound). A 'Muted Neutral' (MN) treatment (five seconds of 'nothing', no CS or US) was used to control for the effect of



168 sound in the other treatments. The sound cues were played from a computer at a sound pressure level  
169 of 75 dBA. Each of the four sound cues was used to signal the presence of each type of US.  
170 Consequently four cue groups of three hens (from the same home pen) each experienced different  
171 combinations of CS and US.

172  
173 **Insert Table 1 here**  
174

### 175 **2.3 Experimental Apparatus**

176 An experimental pen (200cm long x 125cm wide x 60cm high) was located in a sound-proofed room  
177 adjacent to the room in which the birds were housed. The pen contained two compartments of equal  
178 size - a waiting compartment and a reward compartment, separated by a wire-mesh partition and  
179 connected by a swing door in the middle of the partition. The door could be locked and released by  
180 increasing or decreasing an electrical current going through an electromagnet attached to it, and only  
181 opened in the direction of the reward compartment. Three of the four walls of the experimental pen  
182 were made of plywood and one was made of wire-mesh to allow video recordings of both  
183 compartments. A lamp, secured to the middle of the outer wall at 60cm from the floor could be  
184 operated by the experimenter who sat behind a screen out of sight of the hens during tests. This light  
185 shone into the reward compartment and was used to highlight the reward and indicate that the door  
186 was open.

187  
188 The apparatus used for rewards were a white food bowl, a tray filled with topsoil / sand mix, and the  
189 birds' normal feeders. The topsoil/sand mix was chosen after a review of the literature on functional  
190 substrates for dustbathing, and its dry crumbly texture made it an ideal substrate for this purpose  
191 (Olsson and Keeling, 2005). Duplicates of the white food bowl containing food and a white tray filled  
192 with the topsoil /sand mix were put in the home pens three days before training started to allow birds  
193 to become accustomed to them.

### 194 195 **2.4 Training Procedure**

196 The training consisted of several phases similar to those used by Zimmerman et al. (2011) with  
197 adjustments in the length of each phase due to the number of conditioned stimuli used. As a result of  
198 the hens learning to enter the reward compartment more slowly, training took place over 25 days  
199 (compared with 22 days in Zimmerman et al. (2011)). In phase 1 (Days 1 to 7) birds were trained to  
200 use the swing door. This initial training was done in groups of three to increase the speed of training.  
201 During the first two days the door was kept fully open, and a trail of sunflower seeds led through the  
202 door into the reward compartment where the white bowl held more sunflower seeds. Each home pen  
203 group of three birds was allowed to accustom themselves to moving from the waiting compartment to  
204 the reward compartment following the trail of seeds. In the following five days, home pen groups  
205 were trained to go through the door, the opening width of which was gradually reduced more on each  
206 day. During this period, the birds were food deprived for an average of two hours and mealworm were  
207 placed in the white bowl in the reward compartment to incentivise the birds to go through the door.  
208 Each group of three birds experienced eight consecutive trials. On the final day the door was fully  
209 closed, but unlocked, so that the birds had to push through it to gain access to the mealworm.  
210  
211 In phase two (Days 8 to 13), the birds were individually trained to recognise the specific CS for each  
212 of the rewards. The containers containing the dusty substrate were removed from the home pens from  
213 this time. In this phase, the door was kept unlocked and birds were given 10 minutes to go through the  
214 swing door after their particular CS for Mealworm, Dusty substrate, or Normal Food, was played and  
215 the light switched on. All birds entered the compartment within the 10 minute time limit. After  
216 consumption of the reward, the light was switched off and the birds were guided back into waiting  
217 area by the experimenter. Each cue group was trained for one stimulus on one day and each individual  
218 experienced three consecutive trials. Hence, during the six days, each hen experienced six trials of  
219 each stimulus with the door unlocked. In phase 3 (Days 14 – 17), the swing door was locked and the  
220 CS and light signal were made contingent on the behaviour of the bird. Birds were placed individually  
221 into the waiting compartment and allowed to try to push through the locked door twice before the CS  
222 was played and the light switched on at the same time as the door was unlocked. This procedure was

repeated three times and then the CS was played and the light switched on / door unlocked at random when the bird was not near the door. When the bird went through the door immediately after the CS/light was given in five consecutive trials, the training session was ended and birds were returned to their home pen. In phase 4 (Days 18-19), a trace conditioning procedure (Moe et al., 2009) was used to accustom the hens to an interval between the CS being played and activating the light signalling the door was unlocked. The CS was played for five seconds and the interval between the end of the CS and the light signal was gradually increased from 0 to 15 seconds over five consecutive trials for each individual bird on each day. Birds successfully reached our criterion when they went through the swing door within five seconds after the light had been switched on. In phase 5 (Days 20-22), all birds were introduced to their Sound Neutral CS. In these SN trials nothing happened after the light had been switched on. The light was kept on for 15 seconds and then switched off. In phase 6 (Days 23-25), rewarded (Mealworm, Dusty substrate, Normal Food) and SN trials were presented in a randomised order, with each cue being presented at least once to each bird on each day. Birds successfully reached our criterion when they went through the swing door within five seconds on every rewarded trial.

## **2.5 Test Procedure**

For testing, a bird was collected from her home pen and put in the experimental pen. The order of testing was determined using an orthogonal latin square design where every single condition follows another on two occasions. Each hen received one test session per day on five consecutive days. Birds were deprived of food for an average of 1.5 hours prior to testing, and deprived of a substrate suitable for foraging and dustbathing in their home pens for all five days of the test period. Each test session consisted of presentation of each of five stimuli; three reward treatments (Mealworm, Dusty substrate, Normal Food), one SN and one MN trial. At the start of each session a bird was allowed to habituate to the experimental pen for 30 seconds. Then the appropriate CS was given for five seconds, after which behaviour was recorded for 15 seconds before the light was switched on signalling the door was unlocked. There was no CS in the MN trial, but behaviour was recorded for 15 seconds from when the trial started. In the Mealworm trial, after the CS and the 15 second anticipation period, the

door was unlocked and the bird entered the reward compartment and ate the mealworm. Then the light was switched off and the bird was ushered gently into the waiting compartment by the experimenter who held the swing-door open. In the Normal Food trial, the same happened except that the birds were allowed one minute to feed before the light was switched off and the bird was returned to the waiting compartment. In the Dusty substrate trial, the same process was followed except that the birds were allowed to dustbathe or forage (with no food present) for five minutes before the light was switched off and the bird was returned to the waiting compartment. If the birds stopped feeding or foraging / dustbathing and walked away from the stimulus, or engaged in other behaviour in other parts of the pen for a continuous period of 10 seconds, then the trial was ended. In a SN trial, the CS was given and, after a 15 second anticipation period, the light was switched on but the door did not open. In all trials, between the end of the trial and the start of a new waiting period, there was an inter-trial interval of 10, 20 or 30 seconds (balanced between hens), to prevent hens from easily anticipating the start of the next trial,

During the testing procedure video recordings were made using 2 x K-32HCVF, (Kobi, Taiwan) cameras and recorded onto a K9 XQ H.264 DVR (Kobi, Taiwan). These were then transferred to a PC for analysis using Cowlog: Version 2.11 (Hänninen and Pastell, 2009)

## **2.6 Behavioural recording**

**Insert Table 2 here**

The frequencies and durations of selected behaviours (see ethogram, Table 2) were scored from video recordings. The duration of behaviours was recorded from the beginning of the behavioural sequence, until that behaviour ceased. For example, if a bird pecked three times against the door, the duration was measured from the beginning of the first peck until the end of the last peck. Comfort behaviour (as defined by Zimmerman et al.(2011), including preening, wing flapping, feather ruffling, scratching body, yawning and tail wagging) was only infrequently observed during the test periods and therefore was not included in the analysis. Other behaviours omitted from the analysis due to infrequent

occurrence were “Pecking the cage” “Putting head through side mesh” “Scratching the side mesh” “Explore Ground”, “Peck Ground”, “Peck Wall”, “Explore Object” and “Scratch Ground”. For the final analysis, similar behaviours were merged into related groups of behaviour; “Locomotory behaviour” included Walk, Step and Run, and “Motivational behaviour” incorporated Peck Door and Push Door. The other behaviours included in the analysis were “Stand”, and “Alert Head Movements” (see Table 3).

**Insert Table 3 here**

In addition, we measured the following latency time periods for all reward treatments: Time the door was opened to the time the bird entered the reward compartment (Door to Enter), time the door was opened to the time the bird accessed the reward (Door to Reward) and, finally, time the bird entered the reward compartment to the time the bird accessed or consumed the reward (Enter to Reward).

## **2.7 Statistical analysis**

Linear mixed effects models (LMMs) and generalised linear mixed effects models (GLMMs) were performed in R (R Core Team, 2013) using the lme4 (Bates et al., 2015) and glmmADMB (Skaug et al., 2013) packages. Mixed models were used to account for the within-bird variance associated with repeated measurements. Standard statistical models assume independence of residuals, but when measurements are taken from the same individual they are correlated. Mixed effects models allow us to include individual (bird) identity as a random factor, thus enabling us to separate the total variance in the response variable into a within-subject and between-subject variance component. Where LMMs were used, the assumptions of normal distribution, linearity and homoscedasticity of the residuals were checked by visual inspection of residual plots and by Shapiro-Wilks tests. Residuals that deviated from normality were corrected by log transformations. We computed parameter estimates using the maximum likelihood method, and the significance of predictor variables were tested using maximum likelihood ratio tests, (anova function in R). For all LMMs and GLMMs the Chi-squared

statistic ( $\chi^2$ ) and associated P-values are reported. Post hoc analyses were conducted using the lsmeans package (version 2.20-23) in R, applying the Tukey method to adjust *P*-values for multiple comparisons.

The effects of the different treatments on the duration of behaviours during the anticipation period were analysed using LMMs, with each response variable modelled separately. LMMs were also carried out on the three different latency periods. An initial model included Treatment (Dusty substrate, Mealworm, Normal Food, Sound Neutral, Muted Neutral), Cue Group (1-4), Day (1-5), Preceding Treatment (Dusty substrate, Mealworm, Normal Food, Sound Neutral, Muted Neutral, no preceding treatment) and the interaction between Cue Group and Treatment as fixed effects. However, as there were very few significant effects of Day and Preceding Treatment in the model outputs, the data were collapsed to give an average of the duration of each behaviour over the five days. Subsequently, models with the following predictor variables - 1) Treatment only, 2) Treatment and Cue Group and 3) Treatment, Cue Group and Treatment\*Cue Group interaction - were compared using Akaike Information Criterion (AIC) scores and the anova() function, to detect if any models fitted significantly better than the other. The best fitting models, according to AIC scores, are detailed for each response variable in Appendix 1, Table 1 for behavioural responses and Appendix 1, Table 3 for latency periods. In order to meet the assumptions of the linear mixed model, two behavioural variables (Mot D and Alert D) and two latency period variables (Door to Reward and Enter to Reward) were log ( $x+1$ ) transformed. One latency period (Door to Enter) did not meet the assumption of normality of residuals, despite attempts at transformation. We therefore conducted a Friedman test in Minitab 17 (Statistical Software (2010). State College, PA: Minitab, Inc.) with Bird as a blocking factor, and post hoc analyses were performed using two-tailed Wilcoxon Signed Rank tests applying a Bonferroni correction, resulting in a significance level set at  $p=0.02$ .

All behaviour frequency response variables (Stand F, Loco F, Mot F, and Alert F), and the Behavioural Transitions variable, consisted of count data. Poisson models, and other models in the family, may be used to analyse count data and generally require the data to be discrete, whole

numbers. Therefore, we used the original data (not averaged over 5 days) to analyse these variables. Another important assumption of the Poisson distribution is that the mean and the variance of the sample are identical. Stand F and Loco F met this requirement and therefore were analysed using Poisson regression models. When the variance is greater than the mean, (eg. the variance of Mot F was nearly five times greater than its mean), the data is said to be overdispersed which can result in biased standard errors if using a Poisson model. In this case, we used a negative binomial distribution, which accommodates overdispersion. An additional complication, common in count data regression, is having too many zeros, which was the case for 45% of the observations for Mot F. We therefore used a zero-inflated negative binomial model to account for this large amount of zeros. The ‘Behavioural Transitions’ variable contained no zeros, and AIC scores indicated a zero-truncated negative binomial model (type 1) was appropriate for the data. We used the function `glmmADMB` to run all Poisson and negative binomial models.

Model selection for GLMMs was carried out by comparing AIC scores, and by using the `anova()` function, to detect if any models fitted significantly better than the others. Models included the following sets of predictor variables: 1) Treatment only, 2) Treatment and Cue Group, 3) Treatment, Cue Group and Day, 4) Treatment, Cue Group, Day and Preceding Treatment, 5) Treatment, Cue Group, Day and Treatment\*Cue Group Interaction, 6) Treatment, Cue Group and Treatment\*Cue Group Interaction, and 7) Treatment, Cue Group, Day, Preceding Treatment and Treatment\*Cue Group Interaction. Model comparison and AIC scores are detailed in Appendix 1, Table 2. Residual plots were checked by running the models in `lme4` and using the `plot()` function to check for any patterns in the data. Incident rate ratios and 95% confidence levels were extracted, and mean predictions were also checked against observed data to ensure they did not deviate, as deviation would indicate a poorly fitting model.

The variable Alert F was also count data. However, despite initial exploration indicating a Negative Binomial model might be appropriate, the model did not converge. A binomial model was not appropriate due to the fact that birds made no alert head movements during the Dusty substrate

treatment, and therefore one cell contained all zeros. It was decided that removing Dusty substrate from the analysis and running the statistical analysis using the remaining treatment would result in an unacceptable loss of information. Therefore we ran a Friedman test for Alert F with bird as the blocking factor, and post hoc analyses were performed using two-tailed Wilcoxon Signed Rank tests applying a Bonferroni correction, resulting in a significance level set at  $p=0.005$ .

All final model outputs are detailed in Appendix 2. A Bonferroni correction was applied to account for the large number of variables being tested (12 in total) using the same data set, and therefore  $P$ -values of and below 0.004 were considered significant

### **3. RESULTS**

#### **3.1 Effect of signalled rewards compared with neutral treatments**

##### *3.1.1 Behavioural transitions*

All three rewarded sound cues (Dusty substrate, Mealworm and Normal Food) elicited a higher frequency of transitions between behaviours compared with the Muted Neutral (MN) treatment (Table 4 and Fig. 1). The Dusty substrate and Mealworm sound cues also elicited more behavioural transitions than the Sound Neutral (SN) treatment (Table 4 and Fig. 1).

**Insert Fig. 1 here**

##### *3.1.2 “Motivated” behaviour*

Birds also performed significantly higher frequencies and durations of “motivated” behaviour (pushing and pecking at the door) following all three reward sound cues, compared with both the SN and MN treatments (Table 4 and Fig. 2). The frequency of motivated behaviour was also higher following the SN sound cue compared with during the MN treatment (Table 4 and Fig. 2).

Dusty substrate

#### **3.2 Effect of different signalled rewards**



Differential behaviour was elicited by the sound cues signalling the different rewards.

### *3.2.1 Motivated behaviour*

Birds performed “motivated” behaviour (pushing and pecking at the door) significantly more frequently and for a longer duration following the cue signalling the Dusty substrate compared with following the Mealworm and Normal Food sound cues (Table 4 and Fig. 2).

**Insert Figure 2 here**

### *3.2.3 Standing*

Birds stood still for less time in the period following the Dusty substrate sound cue compared with all the other treatments (Table 4 and Appendix 3). The frequency of this behaviour was lower following the Dusty substrate sound cue compared with all other treatments except the MN treatment (Table 4 and Appendix 3). The cue signalling Mealworm led to birds standing still for less time compared with following the SN sound cue (Table 4 and Appendix 3).

### *3.2.4 Latency to access the rewards*

After birds had entered the reward compartment, birds accessed/consumed the Dusty substrate and Mealworm rewards faster than their normal food (Enter – Access Reward), and accessed the Dusty substrate faster than normal food once the door had been opened (Door to Reward) (Table 4 and Fig 3). Treatment had no effect on the time birds took to enter once the door had been opened (Door Open to Enter) (Table 5 and Fig. 3).

**Insert Figure 3 here**

**Insert Table 4 and Table 5 here (landscape orientation)**

### **3.3 Effect of signalled neutral event**

#### *3.3.1 Alert head movements*

Birds exhibited significantly higher durations of alert head movements after the SN treatment had been signalled compared with after the sound cues for all three rewards (Table 4 and Fig. 4). The frequency of alert head movements was significantly higher following the SN sound cue compared to after the Dusty substrate sound cue (Table 5 and Fig.4). (There were no alert head movements recorded during the Dusty substrate treatment).

**Insert Figure 4 here**

#### *3.4 Locomotion*

The frequency and duration of locomotory behaviour were not affected by treatment (Table 4 and Appendix 3).

## **4. DISCUSSION**

The results from our study confirm that hens differentially anticipate food and non-food rewards. Differences in the intensity of behaviours appear to reveal how birds ranked rewards, with the cue signalling the non-food reward (Dusty substrate) consistently inducing higher frequencies and durations of certain behaviours compared with both Mealworm and Normal Food. An increase in behavioural transitions, as opposed to an increase in head movements, appears to characterise the anticipation of all reward types.

### **4.1 Effect of Sound Cues**

Consistent differences in behaviour elicited by the sound cues showed that birds learned to discriminate between cues signalling the imminent arrival of rewards and the cue or absence of a cue signalling a sound neutral or muted neutral treatment where nothing occurred. This confirms findings made by Zimmerman et al., (2011) and Moe et al. (2013) where laying hens were found to differentially anticipate different rewards or events.

## **4.2 Increase in activity / Behavioural transitions**

We predicted that anticipation of rewards would cause an increase in activity, which would suggest “preparatory behaviour” (Spruijt et al., 2001) in order to access the resource. Our results supported this prediction, revealing that birds performed a higher frequency of behavioural transitions in anticipation of the three rewards compared with the neutral treatments. The number of transitions between behaviours is deemed to be a good indicator of activity (van der Harst et al., 2003; Vinke et al., 2004; Anderson et al., 2015), and changes in patterns of goal-directed behaviour are suggested to be an expression of “wanting” (Boissy et al., 2007). Our results appear to contradict previous findings by Moe et al. (2009) in which hens demonstrated a lack of activity more akin to the response shown by cats when a food reward was signalled. An increase in activity in anticipation of rewards in our study brings our findings closer to those relating to many other animals such as rats (Bos et al., 2003), mink (Vinke et al., 2004), horses (Peters et al., 2012), and lambs (Anderson et al., 2015) , and suggests preparation in order to facilitate consumption of the rewards. In the case of our non-food reward, the dusty substrate, this may reflect arousal in anticipation of being able to satisfy a hard-wired need (Wichman and Keeling, 2008).

It is also worth considering, however, that the differences between our findings relating to activity levels and the findings of Moe et al. (2009; 2011; 2013) may lie in the experimental procedure. In our experiment, hens had been trained to expect a reward in a specific location that they were able to see, whereas in experiments conducted by Moe et al. (2009; 2011; 2013) the reward was delivered into the birds’ home pen after a light cue, and the birds had no ability to see the reward or the location of potential rewards beforehand. The increased activity in our experiment may therefore reflect the hens’ motivation to gain access to the reward location after a reward was signalled, rather than wait for a reward to be delivered.

We also note that there is a distinction to be made between behavioural transitions and locomotory behaviour, both of which are indicators of activity. In our experiment, locomotory behaviour was not

significantly affected by treatment. There are many possible reasons for locomotory behaviour in chickens. Zimmerman et al.(2011) found that birds took an increased number of steps in their negative treatment compared with their positive treatment (mealworm), and hypothesised that this could potentially be “pacing” as a result of frustration, but they also suggest that increased locomotion is typical foraging behaviour in chickens. Kostal et al. (1992) also suggested that an increase in walking reflected a motivation to forage in anticipation of food. Hence, the lack of significance between rewards and neutral treatments could be because there were different motivations for locomotion in the different treatment; during the MN and SN treatment, the absence of a reward could have induced frustration leading to pacing, or hens may have been walking or foraging more because they were hungry, whereas in rewarded treatments, locomotion stemmed more from motivation to access the reward. Further investigations are needed to investigate the underlying motivation for locomotory behaviour during anticipation of rewards.

#### **4.3 Behaviour reflects differences between signalled rewards**

The analysis of the behaviours “Motivated behaviour” and “Standing” revealed differences between reward treatments, which confirmed that birds were able to associate the respective sound cues with the different reward types, supporting findings by Zimmerman et al. (2011) that birds were able to learn associations between sound cues and positive, negative and neutral events, and also by Moe et al. (2013) that birds were able to associate two different light cues with two different food rewards. Our findings extend this knowledge, as behaviours in our study revealed differences between food and non-food rewards.

#### **4.4 Behaviour reflects how birds rank rewards**

The behavioural responses of our study appear to provide evidence that hens’ preferences for rewards are ranked. In a study on how food rewards are differentially expressed in hens, Moe et al (2013) suggested that differences in the frequency of head movements reflected the incentive value of mealworm over wholewheat. The authors also suggested a need to investigate whether cue-induced behaviours may be food-reward specific. However, our experiment reveals that there were

quantitative rather than qualitative differences in behaviour between all three rewards, and therefore both food and non-food rewards appear to evoke a general anticipatory response in which the intensity of the behaviour (frequency, duration, etc.) differentiates the ranking of the reward but not the type. .

#### **4.5 Does the anticipation of food elicit a higher intensity of behavioural expression?**

We predicted that cues signalling food rewards would evoke a higher intensity of behavioural expression. Our results confirm that anticipation of different rewards is differentially expressed in the frequency and duration of some behaviours. However, it was the sound cue for Dusty substrate that elicited a higher duration of pushing and pecking at the door and significantly less standing than following the signals of both the other rewards, results which do not support our hypothesis.

If the higher intensity of behaviours induced by the signal for Dusty substrate reflects the hens' greater motivation to access that reward compared with the others, then our results suggest that hens in our experiment ranked the Dusty substrate as more attractive than the food reward. This outcome does not support a previous study by Petherick et al (1993), where motivation to access a dusty substrate after deprivation was lower than motivation to access food. Furthermore, Dawkins (1983) demonstrated that access to litter under restricted time conditions was deemed by hungry birds to be of less value than food. However, in the same study, when birds had not been food restricted, they overwhelmingly chose access to litter over food. In our experiment, birds only had access to a dusty substrate during testing, and although birds in our study were food restricted, the duration of this restriction did not exceed two hours. Therefore, it appears that hens may have felt satiated enough that a Dusty substrate represented a more attractive reward than food. In concurrence with this idea, Widowski and Duncan (2000) proposed that dustbathing is motivated by pleasure, and if there is a low cost of performing dustbathing behaviour, then the fitness benefit increases. Fraser and Duncan (1998) laid out the framework for this idea, suggesting that positive affective or "pleasure" states evolve in "opportunity situations" rather than in "need situations". Therefore, although scientists suggest that dustbathing is important for animal welfare and the incidence of sham dustbathing in battery cages is deemed to be an indicator of high motivation to perform this behaviour (Olsson and

Keeling, 2005), we cannot conclude from our results that the apparent higher motivation to dustbathe rather than access food reflects a higher need to perform this behaviour.

Despite these equivocal results, we can conclude that access to a dusty substrate is an attractive resource which is important to hens, even when birds have not been exposed to this kind of substrate before. Our birds' original home environment was caged, with no access to a substrate for dustbathing. However, most hens performed a full sequence of dustbathing behaviour twice during the five test days. (The sequence consists of scratching and bill raking in the substrate, followed by the bird erecting its feathers, squatting down in the substrate, wing shaking, head rubbing, scratching with one leg, and sidelying or side rubbing in the substrate). Chickens dustbathe every two days on average, however they tend not to dustbathe during the morning hours (Vestergaard, 1982) which highlights the fact that birds in our experiment were highly motivated to perform this behaviour (testing had ended by 12.30pm on each day). If the birds did not perform the full sequence of dustbathing behaviours, they spent the majority their time pecking in the substrate. Scratching, foraging and pecking in litter, as well as being precursors to dustbathing, are behaviours which chickens are motivated to perform in their own right (Olsson and Keeling, 2005). In addition, although no hens appeared to perform nesting behaviour during the trials in our experiment, a dustbathing substrate may also be perceived as a potential nest site in a cage environment. (Smith et al., 1993). Thus, a dusty substrate can be a multi-faceted resource, and in our experiment, may have also represented the opportunity to be "rewarded" for a longer period (as we allowed them access to the container full of substrate for five minutes, as opposed one minute for Normal Food and Mealworm respectively).

We also used the latency of the chickens to access the rewards as a proxy of motivation. The results showed differences between the times taken to access the rewards, and also provide evidence that suggests consistent preferences between the two food rewards. Chickens appeared to consistently rank the Dusty substrate as more attractive than Normal Food, but, once in the reward compartment, they reached the Mealworm reward quickest. This could be due to the fact that the mealworm represented a

live target which could potentially disappear, and the movement of the mealworm incentivised them to consume the reward quickly. The difference in speed of accessing Mealworm compared with the birds' normal pellet feed supports previous findings by Bruce et al (2003), who found that chickens were highly motivated to access mealworm in comparison with five other food items. Davies et al (2014) also found that anticipation of mealworm resulted in increased heart rates and head movements, as well as a faster latency to reach a food bowl.

#### **4.5 Head movements**

In our study, the SN cue elicited significantly more alert head movements than all the reward sound cues and the MN treatment. This finding does not support a previous study conducted by Moe et al. (2011) which suggested that head movements are the most salient indicator of anticipatory behaviour in chickens, having found that these movements were attenuated by a dopamine D2 antagonist. Head movements were also found to be a more sensitive measure of arousal than heart-rate during decision-making in chickens (Davies et al., 2014). However, Zimmerman et al (2011) theorized that head movements imply increased vigilance in anticipation of a negative event after their study revealed hens increased their head movements prior to being squirted with water.

##### *4.5.1 Sound cue effect – context-mediated equivalence?*

The fact that the SN treatment elicited the most alert behaviour (head movements) may indicate some context-mediated equivalence (Molet et al., 2012) where cues that share a common context at different times come to be treated as equivalent. We attempted to use simple sound cues which could be easily differentiated (by humans). However, all sound cues except one indicated a reward, and this contextual information may have been generalised to the sound cue for the SN (no reward) treatment. Moe et al. (2013) experienced a similar result, and suggested that unrewarded blue light (their neutral treatment) may have induced arousal due to context-mediated equivalence, or that hens, to some extent, generalised the stimuli “light” and responded to the unrewarded cue colour. Zimmerman et al. (2011) investigated whether hens could differentiate between cues signalling positive, negative and neutral events. Their results showed that hens did discriminate, but as a result of the increased

attention to one of the cues, the authors suggested that the nature of the cues was paramount; increased attention and head movements were interpreted as resulting from birds attempting to localise the source of the sound. We conclude that both sound and light cues appear to have an arousal effect on hens which induces some types of anticipatory behaviour (alert head movements), whether a reward has been signalled or not.

## **5. Conclusions**

We found that hens expressed behaviour differentially in response to reward type, both food and non-food, and that these differences seemed to reflect the incentive value of the rewards. The frequency and duration of behaviours performed were the defining factors in discriminating between rewards, and therefore behaviour did not appear to be specific to food rewards. When access to a substrate suitable for dustbathing was restricted, and when only slightly food restricted, chickens appeared to rank a dusty substrate as more attractive than food rewards, with mealworm being preferred to normal food, as determined by behavioural responses combined with the latency to access the rewards. Our findings suggest that, when chickens are able to see the reward location, and a cue reliably signals impending delivery of a reward, anticipatory behaviour is expressed in attempts to access the reward location as well as a higher frequency of behavioural transitions, extending the range of behaviour known to be expressed in anticipation of rewards.

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